(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property
Organization
International Bureau

(43) International Publication Date
17 June 2004 (17.06.2004)

PCT

(10) International Publication Number
WO 2004/050905 A1

(51) International Patent Classification[7]: C12Q 1/66, 1/68

(21) International Application Number:
PCT/GB2003/005272

(22) International Filing Date: 2 December 2003 (02.12.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0228139.2   3 December 2002 (03.12.2002)   GB

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(81) Designated States (national): AE, AG, AL, AM, AT (utility model), AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ (utility model), CZ, DE (utility model), DE, DK (utility model), DK, DM, DZ, EC, EE (utility model), EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK (utility model), SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States (regional): ARIPO patent (BW, GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— with international search report
— before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: EUKARYOTIC BIOSENSOR MAKING USE OF A CALCIUM REGULATED LIGHT EMITTING ENZYME

(57) Abstract: The present invention provides a method of using eukaryotic cells being transformed with a light emitting Ca^{2+} regulated photoprotein gene for determining the presence or absence of at least one toxic substance in a sample and for assisting in the identification of the toxicant(s). More specifically there is provided a toxicity assay for various uses including determining the presence of toxins and in particular heavy metals and organophenols, general cytotoxicity testing of pure chemicals and chemical mixtures in particular for drug development testing, testing of food and drink products, cosmetics testing and identification of organisms in particular of fungal strains.

EUKARYOTIC BIOSENSOR MAKING USE OF A CALCIUM REGULATED LIGHT EMITTING ENZYME

1 "Eukaryotic Biosensor"

2

3 The present invention provides a method of using

4 transformed eukaryotic cells or organisms for

5 determining the presence or absence of at least one

6 toxic substance in a sample and for assisting in the

7 identification of the toxicant(s). More

8 specifically there is provided a toxicity assay for

9 various uses including determining the presence of

10 toxins, general cytotoxicity testing of pure

11 chemicals and chemical mixtures in particular for

12 drug development testing, testing of food and drink

13 products, cosmetics testing and identification of

14 organisms in particular of fungal strains

15

16 The release of contaminating substances into an

17 environment such as a waterway or an area of

18 agricultural land can have serious effects on the

19 ecosystems found in that environment. It is

20 important to be able to analyse these effects both

21 prior to the release of such contaminants so as to

2

1 manage their treatment or release, and after release
2 so as to determine and counteract their effects.
3
4 Current methods used to monitor water quality and
5 screen effluent generally involve chemical toxicity
6 tests. However, these tests require a general idea
7 of the type of contaminant being tested for and can
8 be very expensive.
9
10 Similarly the presence of contaminating substances
11 or toxins can be problematic in other areas such as
12 food and drink manufacture and cosmetics
13 manufacture. There are also instances, such as in
14 drug development and cosmetic industry, where the
15 substance of interest i.e. the potential new drug
16 may itself be a contaminating substance or toxin and
17 this needs to be checked.
18
19 Biosensors are used for toxicity testing and are
20 well known in the field. Toxicity depends on a
21 variety of factors including pH, temperature,
22 salinity and contaminant concentration, but depends
23 especially on the test organism used in the sensor.
24
25 One of the most commonly used organisms is the
26 bioluminescent bacterium, Vibrio fischeri. The
27 bioluminescence involved is mediated by the
28 luciferin-luciferase enzyme system wherein light
29 emission is dependent on the electron transfer
30 chain. Any disruption to the electron transfer
31 chain, for example on exposure to a toxicant,
32 affects light emission. Light emission at the time a

3

1   substance is added is therefore indicative of the
2   presence of a toxic substance.
3
4   This system, however, only provides a simple
5   indication of whether a contaminant is toxic or not.
6   No detailed information is obtained on how toxic the
7   contaminant is, nor is the contaminant identified.
8
9   The terms toxicant and toxin as herein described
10 relate to compounds, chemicals and mixtures of
11 chemicals which have an effect on eukaryotic cells
12 or organisms and in particular which are toxic to
13 eukaryotic organisms such as fungus or which have
14 anti-fungal activity.
15
16 The term eukaryote as herein described relates to
17 eukaryotic cells or organisms.
18
19 According to a first aspect of the present invention
20 there is provided a method of determining the
21 presence of a toxicant in a test sample, comprising
22 the steps of;
23   -   exposing a eukaryote that has been
24   transformed with a light emitting Ca^{2+}
25   regulated photoprotein gene to a test sample
26   -   measuring the light produced by the
27   transformed cell/organism
28   -   determining whether the amount of light is
29   above or below a defined threshold at the
30   time of exposure.
31
32

4

1   Optionally the eukaryote is a fungi.

2   (throughout this document fungi should be considered

3   under its typical classification as covering both

4   multicellular organisms and unicellular organisms

5   such as the yeast *Saccharomyces cerviseae*

6   Preferably the fungi is a filamentous fungi.

7

8   More preferably the fungi is of the *Aspergillus*

9   species.

10

11

12   Alternatively the eukaryote is a mammalian cell.

13

14   A further alternative is that the eukaryote is a

15   plant cell.

16

17   Preferably the test sample comprises a toxicant.

18

19   Preferably the light emitting Ca^{2+} regulated

20   photoprotein gene is a recombinant gene.

21

22   Preferably the light emitting Ca^{2+} regulated

23   photoprotein gene is selected from the group

24   comprising;

25   - aequorin gene

26   - halistaurin (mitrocomin) gene

27   - phialidin (clytin) gene

28   - obelin gene

29   - mnemiopsin gene

30   - berovin gene

31

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5

1   Optionally, the light emitting Ca^{2+} regulated

2   photoprotein gene may be a functional homologue of a

3   gene selected from the group comprising;

4   - aequorin gene

5   - halistaurin (mitrocomin) gene

6   - phialidin (clytin) gene

7   - obelin gene

8   - mnemiopsin gene

9   - berovin gene

10

11   Most preferably the light emitting Ca^{2+} regulated

12   photoprotein gene is an aequorin gene.

13

14   More preferably the light emitting Ca^{2+} regulated

15   photoprotein gene is a recombinant aequorin gene.

16

17   Preferably the light that is measured is in the form

18   of luminescence.

19

20   Optionally the test sample is added in advance of

21   the application of a stimulus to the test sample.

22

23   Preferably the stimulus is at least one or more from

24   the group comprising; mechanical perturbation, hypo-

25   osmotic shock, and change in external calcium

26   chloride concentration, temperature shock, pH shock.

27

28   Preferably the test sample is added 1 minute to 1

29   hour prior to the application of the stimulus.

30

31   More preferably the test sample is added 5 minutes

32   prior to the application of the stimulus.

6

1

2   More preferably the test sample is added 30 minutes

3   prior to the application of the stimulus.

4

5   According to a second aspect of the present

6   invention there is provided a method of determining

7   the presence of a toxicant in a test sample,

8   comprising the steps of;

9   - exposing a eukaryote that has been

10   transformed with a light emitting Ca^{2+}

11   regulated photoprotein gene to a test sample

12   - measuring the light produced by the

13   transformed cell/organism

14   - determining whether the amount of light is

15   above a defined threshold at a specified

16   time after the time of exposure.

17

18   Optionally the method comprises the step of

19   determining whether the amount of light is below a

20   defined threshold.

21

22   Optionally the specified time after the time of

23   exposure is 11 minutes.

24

25   Optionally the eukaryote is a fungi.

26

27   Preferably the fungi is a filamentous fungi.

28

29   More preferably the fungi is of the *Aspergillus*

30   species.

31

32

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7

1   Alternatively the eukaryote is a mammalian cell.

2

3   A further alternative is that the eukaryote is a

4   plant cell.

5

6   Preferably the test sample comprises a toxicant.

7

8   Preferably the light emitting Ca^{2+} regulated

9   photoprotein gene is a recombinant gene.

10

11   Preferably the light emitting Ca^{2+} regulated

12   photoprotein gene is selected from the group

13   comprising;

14   - aequorin gene

15   - halistaurin (mitrocomin) gene

16   - phialidin (clytin) gene

17   - obelin gene

18   - mnemiopsin gene

19   - berovin gene

20

21   Optionally, the light emitting Ca^{2+} regulated

22   photoprotein gene may be a functional homologue of a

23   gene selected from the group comprising;

24   - aequorin gene

25   - halistaurin (mitrocomin) gene

26   - phialidin (clytin) gene

27   - obelin gene

28   - mnemiopsin gene

29   - berovin gene

30

8

1 Most preferably the light emitting Ca^{2+} regulated
2 photoprotein gene is an aequorin gene.
3
4 More preferably the light emitting Ca^{2+} regulated
5 photoprotein gene is a recombinant aequorin gene.
6
7 Preferably the light that is measured is in the form
8 of luminescence.
9
10 Optionally the test sample is added in advance of
11 the application of a stimulus to the test sample.
12
13 Preferably the stimulus is at least one or more from
14 the group comprising; mechanical perturbation, hypo-
15 osmotic shock, change in external calcium chloride
16 concentration, temperature shock, pH shock.
17
18 Preferably the test sample is added 1 minute to 1
19 hour prior to the application of the stimulus.
20
21 More preferably the test sample is added 5 minutes
22 prior to the application of the stimulus.
23
24 More preferably the test sample is added 30 minutes
25 prior to the application of the stimulus.
26
27 According to a third aspect of the present invention
28 there is provided a method of determining the
29 presence of a toxicant in a test sample, comprising
30 the steps of;

9

1   - exposing a eukaryote that has been

2   transformed with a light emitting Ca^{2+}

3   regulated photoprotein gene to a test sample

4   - measuring the light produced by the

5   transformed cell/organism

6   - and comparing at least one parameter of the

7   light measurement data with a bank of known

8   toxicity reference data.

9

10   Optionally the method comprises the step of

11   determining whether the amount of light is below a

12   defined threshold.

13

14   Optionally the specified time after the time of

15   exposure is 11 minutes.

16

17   Optionally the eukaryote is a fungi.

18

19   Preferably the fungi is a filamentous fungi.

20

21   More preferably the fungi is of the *Aspergillus*

22   species.

23   Do we need next two sentences

24   Most preferably the fungi is *Aspergillus awamori.*

25

26   Most preferably the strain of *Aspergillus awamori* is

27   strain 66A.

28

29   Alternatively the eukaryote is a mammalian cell.

30

31   A further alternative is that the eukaryote is a

32   plant cell.

10

1

2   Preferably the test sample comprises a toxicant.

3

4   Preferably the light emitting Ca^{2+} regulated
5   photoprotein gene is a recombinant gene.

6

7   Preferably the light emitting Ca^{2+} regulated
8   photoprotein gene is selected from the group
9   comprising;
10   - aequorin gene
11   - halistaurin (mitrocomin) gene
12   - phialidin (clytin) gene
13   - obelin gene
14   - mnemiopsin gene
15   - berovin gene

16

17   Optionally, the light emitting Ca^{2+} regulated
18   photoprotein gene may be a functional homologue of a
19   gene selected from the group comprising;
20   - aequorin gene
21   - halistaurin (mitrocomin) gene
22   - phialidin (clytin) gene
23   - obelin gene
24   - mnemiopsin gene
25   - berovin gene

26

27   Most preferably the light emitting Ca^{2+} regulated
28   photoprotein gene is an aequorin gene.

29

30   More preferably the light emitting Ca^{2+} regulated
31   photoprotein gene is a recombinant aequorin gene.

11

1

2   Preferably the light that is measured is in the form

3   of luminescence.

4

5   Optionally the test sample is added in advance of

6   the application of a stimulus to the test sample.

7

8   Preferably the stimulus is at least one or more from

9   the group comprising; mechanical perturbation, hypo-

10   osmotic shock, change in external calcium chloride

11   concentration, temperture shock, pH shock.

12

13   Preferably the test sample is added 1 minute to 1

14   hour prior to the application of the stimulus.

15

16   More preferably the test sample is added 5 minutes

17   prior to the application of the stimulus.

18

19   More preferably the test sample is added 30 minutes

20   prior to the application of the stimulus.

21

22   Preferably, the method is used to determine the

23   amount of toxicant in the sample.

24

25   Optionally, the method is used to identify the

26   toxicant in the sample.

27

28   According to a fourth aspect of the present

29   invention there is provided a method of determining

30   the presence of a toxicant in a test sample,

31   comprising the steps of;

12

1   - exposing a eukaryote that has been

2   transformed with a light emitting Ca^{2+}

3   regulated photoprotein gene to a test sample

4   - measuring the light produced by the

5   transformed cell/organism

6   - converting the light data into a cytosolic

7   free calcium ion concentration trace,

8   - and comparing at least one parameter of the

9   cytosolic free calcium ion concentration

10   trace with a bank of known toxicity

11   reference data.

12

13 Optionally the method comprises the step of

14 determining whether the amount of light is below a

15 defined threshold.

16

17 Optionally the specified time after the time of

18 exposure is 11 minutes.

19

20 Optionally the eukaryote is a fungi.

21

22 Preferably the fungi is a filamentous fungi.

23

24 More preferably the fungi is of the *Aspergillus*

25 species.

26

27

28 Alternatively the eukaryote is a mammalian cell.

29

30 A further alternative is that the eukaryote is a

31 plant cell.

32

13

1   Preferably the test sample comprises a toxicant.

2

3   Preferably the light emitting Ca^{2+} regulated
4   photoprotein gene is a recombinant gene.

5

6   Preferably the light emitting Ca^{2+} regulated
7   photoprotein gene is selected from the group
8   comprising;
9   - aequorin gene
10   - halistaurin (mitrocomin) gene
11   - phialidin (clytin) gene
12   - obelin gene
13   - mnemiopsin gene
14   - berovin gene

15

16 Optionally, the light emitting Ca^{2+} regulated
17 photoprotein gene may be a functional homologue of a
18 gene selected from the group comprising;
19   - aequorin gene
20   - halistaurin (mitrocomin) gene
21   - phialidin (clytin) gene
22   - obelin gene
23   - mnemiopsin gene
24   - berovin gene

25

26 Most preferably the light emitting Ca^{2+} regulated
27 photoprotein gene is an aequorin gene.

28

29 More preferably the light emitting Ca^{2+} regulated
30 photoprotein gene is a recombinant aequorin gene.

31

14

1 Preferably the light that is measured is in the form
2 of luminescence.
3
4 Optionally the test sample is added in advance of
5 the application of a stimulus to the test sample.
6
7 Preferably the stimulus is at least one or more from
8 the group comprising; mechanical perturbation, hypo-
9 osmotic shock, change in external calcium chloride
10 concentration, temperature shock, pH shock.
11
12 Preferably the test sample is added 1 minute to 1
13 hour prior to the application of the stimulus.
14
15 More preferably the test sample is added 5 minutes
16 prior to the application of the stimulus.
17
18 More preferably the test sample is added 30 minutes
19 prior to the application of the stimulus.
20 Preferably light is measured for between 1 minute
21 and 5 hours following the application of the
22 stimulus.
23
24 More preferably light is measured for 5 minutes
25 following the application of the stimulus.
26
27 Preferably, the cytosolic free calcium ion trace is
28 a plot of the cytosolic free calcium ion
29 concentration against time.
30
31 Preferably the parameter is at least one or more
32 selected from the group comprising;

15

1   - lag time

2   - rise time

3   - absolute amplitude

4   - relative amplitude

5   - Length of transient

6   - number of cytosolic free calcium ion

7   concentration increases

8   - percentage increase in final cytosolic free

9   calcium ion concentration resting level

10   - percentage increase in recovery time

11   - percentage increase in pre-stimulating

12   cytosolic free calcium ion concentration

13   resting level

14   - Total concentration of Ca^{2+} released.

15

16   Preferably, the method is used to determine the

17   amount of toxicant in the sample.

18

19   Optionally, the method is used to identify the

20   toxicant in the sample.

21

22   According to a fifth aspect of the present invention

23   there is provided an assay for use in determining

24   the presence of a known toxicant in a test sample,

25   the assay comprising the steps of;

26   - exposing a fungi transformed with a

27   recombinant aequorin gene to a test sample of

28   a substance,

29

30   - measuring the luminescence produced by the

31   fungi,

16

1

2   - converting the luminescence data into a

3   cytosolic free calcium ion concentration

4   trace,

5

6   - and comparing at least one parameter of the

7   cytosolic free calcium ion concentration

8   trace with a bank of known toxicity reference

9   data.

10

11   Preferably the cytosolic free calcium ion trace is a

12   plot of the cytosolic free calcium ion concentration

13   against time.

14

15   Preferably the fungi transformed with a recombinant

16   aequorin gene is a filamentous fungi.

17

18   More preferably the fungi is of the *Aspergillus*

19   species.

20

21

22   Preferably the substance is a contaminant.

23

24   Preferably the substance is a contaminated sample.

25

26   Preferably the parameter is at least one or more

27   selected from the group comprising; lag time, rise

28   time, absolute amplitude, relative amplitude, length

29   of transient at 20%, 50% and 80% of maximum

30   amplitude , number of cytosolic free calcium ion

31   concentration increases, percentage increase in

32   final cytosolic free calcium ion concentration

17

1   resting level, percentage increase in recovery time

2   and percentage increase in the total amount of Ca^{2+}

3   released.

4

5   Optionally, the test sample is added in advance of

6   the application of a stimulus to the test sample.

7

8   Preferably the stimulus is at least one or more from

9   the group comprising; mechanical perturbation, hypo-

10   osmotic shock, change in external calcium chloride

11   concentration, temperature shock and pH shock.

12

13   Preferably the test sample is added 1 minute to 1

14   hour prior to the application of the stimulus.

15

16   More preferably the test sample is added 5 minutes

17   prior to the application of the stimulus.

18

19   More preferably the test sample is added 30 minutes

20   prior to the application of the stimulus.

21

22   In such instances, the parameters may include at

23   least one or more selected from the group

24   comprising; lag time, rise time, absolute amplitude,

25   relative amplitude Length of transient at 20%, 50%

26   and 80% of maximum amplitude, number of cytosolic

27   free calcium ion concentration increases, percentage

28   increase in final cytosolic free calcium ion

29   concentration resting level, percentage increase in

30   recovery time, percentage increase in pre-

31   stimulating cytosolic free calcium ion concentration

18

1   resting level and percentage increase in the total
2   amount of Ca^{2+} released.

3

4   Preferably luminescence is measured for between 1
5   minute and 5 hours following the application of the
6   stimulus.

7

8   More preferably luminescence is measured for 5
9   minutes following the application of the stimulus.

10

11 Preferably, the method is used to determine the
12 amount of toxicant in the sample.

13

14 Optionally, the method is used to identify the
15 toxicant in the sample.

16

17 In order to further explain the present invention
18 details of a number of experiments are provided.

19

20 A first experiment comprises testing the effect of
21 pre-incubation of *Aspergillus awamori* with toxicants
22 on cytosolic free calcium ion concentration response
23 to an increase in external calcium chloride.

24

25 A further set of experiments described herein shows
26 attempts to obtain characteristic data for a range
27 of different toxicants at a number of different
28 concentrations. The results demonstrate that each
29 toxicant at each concentration produces a
30 distinctive cytosolic free calcium ion concentration
31 trace whose traits could be used to identify and
32 characterise a toxicant present in a test sample.

19

1

2 A final experiment attempts to determine whether it

3 is possible to identify and characterise individual

4 toxicants from testing samples of mixtures of

5 toxicants in different proportions. The traces

6 produced are distinct for each mixture.

7

8 These results show that it is possible to

9 characterise and identify a specific toxicant from a

10 test sample by using the characteristic data

11 obtained from a cytosolic free calcium ion

12 concentration trace.

13

14 It is also possible to characterise and identify a

15 specific toxicant from a test sample by using the

16 characteristic data obtained from light readings.

17 The main difference between doing light emission and

18 cytosolic free calcium ion concentrations is the

19 removing the step of converting the luminescence

20 data into a cytosolic free calcium ion concentration

21 trace".

22

23 So the method is:

24

25 An assay for use in determining the presence of a

26 known toxicant in a test sample, the assay

27 comprising the steps of;

28   - exposing a fungi transformed with a

29   recombinant aequorin gene to a test sample of

30   a substance,

31   - measuring the luminescence produced by the

32   fungi in relative light units (RLU),

20

1   - and calculating the following parameters: lag
2   time, rise time, length of transient
3   ($LT_{20}, LT_{50}, LT_{80}$), absolute amplitude, relative
4   amplitude, recover time, final level of
5   luminescence, initial level of luminescence,
6   total luminescence.
7

8   Since RLU are not normalised with regard to the
9   biomass, the parameters measured in relative light
10   units (RLU) are different from the cytosolic free
11   calcium ion concentration $[Ca^{2+}]$. Figs 24 and 25 show
12   that the decrease in amplitude caused by 260 mg/l
13   Cr^{6+} is 75% in RLU, and only 65% in Ca^{2+}
14   concentration. Other parameters would differ in a
15   similar way.
16

17   Most of the toxicity testing for environmental
18   pollutants is usually carried out using RLU and
19   therefore the light-emitting essay would be
20   particularly helpful if used alongside other
21   existing biosensors.
22

23   The parameters referred to herein relate to the
24   following;
25

26   Lag Time, the time from addition of the test sample
27   to the time when the cytosolic free calcium ion
28   concentration, $[Ca^{2+}]_c$, began to rise;
29

30   Rise Time, the time from addition of the test sample
31   to the time at which maximum $[Ca^{2+}]_c$ was reached;
32

21

Number of $[Ca^{2+}]_c$ Rises, the number of peaks in $[Ca^{2+}]_c$;

Percentage Increase in Final $[Ca^{2+}]_c$ Resting Level, the percentage increase in resting $[Ca^{2+}]_c$ at the end of the experiment, where the control value is taken to be 100%;

Percentage Increase in Recovery Time, percentage increase in recovery time where recovery time represents the total amount of $[Ca^{2+}]_c$ released during the period of time from the point when the maximum amplitude following calcium chloride treatment was achieved to the point when the $[Ca^{2+}]_c$ reached its final resting level. Recovery time was initially calculated for control cultures. In the control this period of time was calculated as 250 seconds. For the cultures subjected to the treatment with toxicant(s) the total amount of $[Ca^{2+}]_c$ was calculated for the same period of 250 seconds starting from the maximum amplitude. The recovery time of the control cultures was therefore:

$$\frac{\text{total amount of } [Ca^{2+}]_c \ (\mu M) \text{ for the toxicant-treated samples over 250 seconds} \times 100}{\text{total amount of } [Ca^{2+}]_c \ (\mu M) \text{ for the control sample over 250 seconds}}$$

Percentage Increase in pre-Stimulating $[Ca^{2+}]_c$ Resting Level, the percentage increase in $[Ca^{2+}]_c$

22

1   prior to the stimulus, where the control value is
2   taken to be 100%.
3
4   Percentage change in total amount of calcium
5   released during the transient at stage 1 –
6   calculated by integration of the all luminescence
7   obtained after addition of the compounds of interest
8   before subsequent stimulation with physico-chemical
9   stimuli.
10
11   Percentage change in total amount of calcium
12   released during the transient at stage 2 –
13   calculated by integration of the all luminescence
14   obtained after the fungus is stimulated with one of
15   the physico-chemical stimuli.
16
17   Percentage change in total amount of calcium
18   released during the whole transient – calculated by
19   integration of the all luminescence obtained during
20   the period of experiment.
21
22   Length of transient (LT) – this parameter describes
23   the length of the transient when the amplitude of
24   the response is equal a certain percentage from the
25   maximum amplitude.
26   LT_{20} (Length of transient at Amplitude=20% of maximum
27   Amplitude)
28   LT_{50} (Length of transient at Amplitude=50% of maximum
29   Amplitude)
30   LT_{80} (Length of transient at Amplitude=80% of maximum
31   Amplitude)
32

23

1   All secondary increases have to be analysed by the
2   same parameters as primary increases during stages 1
3   and 2.
4   E.g. Amplitude, length, rise time, lag time,
5
6   Percentage change in amplitude should be assessed as
7   the absolute value from point 0 (A_a) and as the
8   relative value from the initial resting level (A_r).
9   The relative changes assess the ability of of the
10   eukaryote to respond to the physiological stimuli.
11   This parameter is important to assess the
12   physiological state of the eukaryote.
13   There is also the possibility of combining one or
14   more of these parameters to obtain further values
15   which can be used for identification of the
16   toxicants in the mixture. For example, the
17   summation of amplitude and recovery time will give
18   the value of total cytosolic free calcium ions
19   emitted from the time when $[Ca^{2+}]_c$ reaches its peak.
20   Also summation of lag time and rise time will give
21   the total time required for $[Ca^{2+}]_c$ to reach its
22   peak. The division of final $[Ca^{2+}]_c$ resting level
23   onto the pre-stimulation $[Ca^{2+}]_c$ resting level will
24   show how many times the $[Ca^{2+}]_c$ resting level has
25   changed after stimulation. Similarly, a division of
26   the final $[Ca^{2+}]_c$ resting level onto the initial
27   $[Ca^{2+}]_c$ resting level prior to the addition of
28   toxicant(s) gives further identifying data.
29   Additionally, the summation of all the data points
30   of the trace gives the total amount of cytosolic
31   free calcium ions released during the monitoring
32   period.

24

1   As mammalian cells are more complex than other

2   eukaryotes such as fungi or plants typically more

3   parameters will be considered.

4

5   The present invention will now be described with

6   reference to the following non-limiting examples and

7   with reference to the figures, wherein:

8

9   Figure 1 shows the characteristic $[Ca^{2+}]_c$ trace

10   produced on addition of 5mM external $CaCl_2$,

11   following a 5 minute pre-incubation with

12   different concentrations of 3,5-DCP.

13

14   Figure 2 shows the characteristic $[Ca^{2+}]_c$ trace

15   produced on addition of 5mM external $CaCl_2$,

16   following a 5 minute pre-incubation with

17   different concentrations of Cr^{6+}.

18

19   Figure 3 shows the characteristic $[Ca^{2+}]_c$ trace

20   produced on addition of 5mM external $CaCl_2$,

21   following a 5 minute pre-incubation with

22   different concentrations of Zn^{2+}.

23

24   Figure 4 shows the characteristic $[Ca^{2+}]_c$ trace

25   produced on addition of 5mM external $CaCl_2$,

26   following a 30 minute pre-incubation with

27   different concentrations of 3,5-DCP.

28

29   Figure 5 shows the characteristic $[Ca^{2+}]_c$ trace

30   produced on addition of 5mM external $CaCl_2$,

25

1   following a 30 minute pre-incubation with

2   different concentrations of Cr^{6+}.

3

4   Figure 6 shows the characteristic $[Ca^{2+}]_c$ trace

5   produced on addition of 5mM external $CaCl_2$,

6   following a 30 minute pre-incubation with

7   different concentrations of Zn^{2+}.

8

9   Figure 7 shows the characteristic cytosolic free

10   calcium ion concentration, $[Ca^{2+}]_c$, trace

11   produced on addition of 5mM $CaCl_2$ following a 5

12   minute pre-incubation with different

13   concentrations of 3,5-dichlorophenol, 3,5-DCP.

14

15   Figure 8 shows the characteristic $[Ca^{2+}]_c$ trace

16   produced on addition of 5mM $CaCl_2$, following a

17   30 minute pre-incubation with different

18   concentrations of 3,5-DCP.

19

20   Figure 9 shows the characteristic $[Ca^{2+}]_c$ trace

21   produced on addition of 5mM $CaCl_2$, following a 5

22   minute pre-incubation with different

23   concentrations of chromium ions, Cr^{6+}.

24

25   Figure 10 shows the characteristic $[Ca^{2+}]_c$ trace

26   produced on addition of 5mM $CaCl_2$, following a

27   30 minute pre-incubation with different

28   concentrations of chromium ions, Cr^{6+}.

29

30   Figure 11 shows the characteristic $[Ca^{2+}]_c$ trace

31   produced on addition of 5mM $CaCl_2$, following a 5

26

1   minute pre-incubation with different

2   concentrations of zinc ions, Zn^{2+}.

3

4   Figure 12 shows the characteristic $[Ca^{2+}]_c$ trace

5   produced on addition of 5mM $CaCl_2$, following a

6   30 minute pre-incubation with different

7   concentrations of zinc ions, Zn^{2+}.

8

9   Figure 13 shows the values of $[Ca^{2+}]_c$ trace

10   parameters characteristic for different

11   concentrations of pentochlorophenol, PCP; sodium

12   dodecyl sulphate, SDS; and Toluene. Parameters

13   assessed are Lag Time, LT; Rise Time, RT;

14   Amplitude, A; Length of transient, LT50;

15   Percentage Increase in pre-Stimulating $[Ca^{2+}]_c$

16   Resting Level, %IpreSRL; Percentage Increase in

17   Final $[Ca^{2+}]_c$ Resting Level, %IFRL; Percentage

18   Increase in Recovery Time, %IRT; and Number of

19   $[Ca^{2+}]_c$ Increases.

20

21   Figure 14 shows the values of $[Ca^{2+}]_c$ trace

22   parameters characteristic for 3,5-DCP, PCP, Zn^{2+},

23   Cr^{6+}, Toluene, and SDS. Parameters assessed are

24   Lag Time, LT; Rise Time, RT; Amplitude, A;

25   Length of transient, LT50; Percentage Increase

26   in pre-Stimulating $[Ca^{2+}]_c$ Resting Level,

27   %IpreSRL; Percentage Increase in Final $[Ca^{2+}]_c$

28   Resting Level, %IFRL; Percentage Increase in

29   Recovery Time, %IRT; and Number of $[Ca^{2+}]_c$

30   Increases.

31

27

1   Figure 15 shows the values of $[Ca^{2+}]_c$ trace

2   parameters characteristic for different mixtures

3   of toxicants. Parameters assessed are Lag Time,

4   LT; Rise Time, RT; Amplitude, A; Length of

5   transient, LT50; Percentage Increase in pre-

6   Stimulating $[Ca^{2+}]_c$ Resting Level, %IpreSRL;

7   Percentage Increase in Final $[Ca^{2+}]_c$ Resting

8   Level, %IFRL; Percentage Increase in Recovery

9   Time, %IRT; and Number of $[Ca^{2+}]_c$ Increases.

10

11   **Effect of pre-incubation of *Aspergillus awamori* with**

12   **toxicants on $[Ca^{2+}]_c$ response to external calcium**

13   **chloride**

14

15   12 ml of sterile VS medium was inoculated with 1 x

16   10^5 spores per ml *A. awamori* strain 66A. 100 µl of

17   the inoculated medium was added to each well of a

18   96-well plate and cultured in a humidity chamber in

19   the presence of free water at 30 °C for 24 hours.

20

21   The following toxicants were tested: 3,5-

22   dichlorophenol, zinc sulphate, and potassium

23   dichromate. Each toxicant was added in a total

24   volume of 25 µl VS medium or water 5 or 30 minutes

25   before addition of 5 mM calcium chloride.

26

27   Luminescence was monitored for 5 minutes following

28   addition of $CaCl_2$. Aequorin was completely

29   discharged by adding 3M calcium chloride in 20%

30   ethanol. The total concentration is thus 1.5 M

31   calcium chloride in 10% ethanol.

32

28

1   Luminometry was performed using an EG & G Berthold
2   (Bad Wildbad, Germany) LB96P Microlumat luminometer.
3   Luminescence data was converted from real light
4   units to $[Ca^{2+}]_c$ values using the following equation:
5
6   PCa = 0.332588 (-log k) + 5.5593,
7
8   where k = luminescence counts per second/total
9   luminescence counts. Total luminescence is measured
10 as an integral of all luminescence up to complete
11 aequorin discharge.
12
13 The Equation is first described in Fricker, M.D.,
14 Plieth, C., Knight, H., Blancaflor, E., Knight,
15 M.R., White, N.S., and Gilroy, S. 1999. Fluorescence
16 and Luminescence Techniques to Probe Ion Activities
17 in Living Plant Cells. In Mason, W.T., editor,
18 *Fluorescent and Luminescent Probes*. Academic Press.
19 London. pp. 569-596.
20 The following parameters were assessed:
21 Rise Time, Amplitude, Length of transient, LT50 and
22 Final $[Ca^{2+}]_c$ Resting Level.
23
24 **Effects of different concentrations of toxicants on**
25 **$[Ca^{2+}]_c$ traces**
26
27 *Aspergillus awamori* were transformed with an
28 expression vector (pAEQ1-15) comprising a gene for
29 synthetic apoaequorin (*aeqS*) under the control of
30 the constitutive glucose-6-phosphate dehydrogenase
31 promoter (*gpdA*).
32

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29

1   These transformants were cultured in 100 µl of

2   Vogel's medium with 1% sucrose (VS medium) in

3   microwell plates for 24 hours before addition of a

4   toxicant or a control of distilled water. Toxicants

5   were dissolved in water to give the concentrations

6   shown below. 25 µl of the each of the following

7   concentrations were added to each culture:

8

TOXICANT	CONCENTRATIONS (mg/l)
3,5-dichlorophenol (3,5-DCP)	0.112, 11.2, 112
Chromium ions (Cr^{6+})	15, 120, 260
Zinc ions (Zn^{2+})	180, 350, 700, 1300

9

10   The cultures were incubated for 5 or 30 minutes

11   before addition of 100 µl 5mM $CaCl_2$. Luminescence

12   was measured for 5 minutes using a plate

13   luminometer. Luminescence data was manually

14   converted from relative light units to cytosolic

15   free calcium ion concentration, $[Ca^{2+}]_c$. This was

16   then plotted against time and parameters of this

17   trace were analysed. Parameters assessed were as

18   follows:

19

20   Rise Time, the time from addition of $CaCl_2$ to the

21   moment when maximum $[Ca^{2+}]_c$ was achieved;

22   Amplitude, the maximum $[Ca^{2+}]_c$ reached during the

23   experiment;

24   Length of transient, at 50% of maximum amplitude the

25   width of the transient at the point where the

26   amplitude equals half of the maximum amplitude of

27   the transient;

30

1　and Final Resting $[Ca^{2+}]_c$ Level, the resting $[Ca^{2+}]_c$

2　at the end of the experiment.

3

4　**Effects of further toxicants on $[Ca^{2+}]_c$ traces**

5

6　Cultures of *Aspergillus awamori* as described above

7　were used to test the effects of further toxicants.

8　The concentrations of toxicants tested were made up

9　as follows in water, where the concentrations tested

10　were based on Dutch target and intervention values

11　for toxicants and Kelly Guidelines for the

12　classification of contaminated soils:

13

TOXICANT	CONCENTRATION (mg/l)
Pentochlorophenol, PCP	0.01, 0.1, 1, 5, 10
Sodium dodecyl sulphate, SDS	1, 10, 50, 100, 500
Toluene	1, 25
3,5-DCP	10
Zn^{2+}	700
Cr^{6+}	15

14

15

16　In the first set-up (S1), 100 µl of each toxicant

17　concentration or of the control (VS medium) were

18　added to the cultures through built-in injectors and

19　luminescence monitored for 5 minutes. In a second

20　set of experiments (S2), cultures were pre-incubated

21　with the toxicant or control for 5 minutes before

22　addition of 5mM $CaCl_2$ in a total volume of 25 µl

23　distilled water (pre-incubation can be anywhere

24　between 1 minute and 96 hours). Luminescence was

31

1   monitored for 5 minutes following addition of $CaCl_2$.

2   (monitoring can be anywhere between 1 minute and 96

3   hours). Luminescence data was converted from

4   relative light units to $[Ca^{2+}]_c$ values as described

5   above. The following parameters were assessed in

6   S1:

7   Lag Time, the time from addition of $CaCl_2$ to the

8   time when $[Ca^{2+}]_c$ began to rise;

9   Rise Time;

10   Absolute amplitude;

11   Relative amplitude

12   Length of transient (LT20, LT50, LT80);

13   Percentage Increase in Final $[Ca^{2+}]_c$ Resting Level,

14   where the control value was taken to be 100%;

15   Percentage Increase in Recovery Time, where the

16   control value was taken to be 100%;

17   and Number of $[Ca^{2+}]_c$ Increases, the number of $[Ca^{2+}]_c$

18   transients.

19   Total Ca^{2+} concentration

20

21   In S2, the Percentage Increase in pre-Stimulating

22   $[Ca^{2+}]_c$ Resting Level, where the control value was

23   taken to be 100%, was assessed in addition to all of

24   the parameters tested in S1.

25

26   **Effects of mixtures containing different proportions**

27   **of toxicants on $[Ca^{2+}]_c$ traces**

28

29   The experiments described when examining the effects

30   of further toxicants were repeated for different

31   mixtures of toxicants. The following mixtures were

32   made up in water for testing:

32

1

2   6 mg/l 3,5-DCP + 12 mg/l Cr^{6+}

3   30 mg/l Cr^{6+} + 350 mg/l Zn^{2+}

4   10 mg/l 3,5-DCP + 350 mg/l Zn^{2+}

5   6 mg/l 3,5-DCP + 12 mg/l Cr^{6+} + 350 mg/l Zn^{2+}

6   Mixture 1:   20 mg/l Cadmium

7   100 mg/l Copper

8   50 mg/l Chromium

9   250 mg/l Zinc

10   500 mg/l SDS

11   Mixture 2:   20 mg/l Cadmium

12   100 mg/l Copper

13   50 mg/l Chromium

14   250 mg/l Zinc

15

16   These experiments demonstrate a novel finding that

17   each toxicant results in a different and

18   characteristic $[Ca^{2+}]_c$ transient. Additionally each

19   concentration of toxicant produces a unique $[Ca^{2+}]_c$

20   transient. From these characteristic fingerprint

21   responses a profile of data can be built up and used

22   to create a bank of data for each toxicant. Results

23   from testing samples can be compared with this data

24   bank and the presence of a particular toxicant can

25   thus be determined. Furthermore, details such as

26   the mode of action of the toxicant, and the amount

27   of toxicant present can be deduced from a comparison

28   with the bank of pre-gathered data.

29

30   **Examples of types of testing that can be carried out**

31   **according to the present invention**

32

33

1   Specific examples of types of test that can be

2   carried out according to the present invention are

3   given below. Although the tests below describe the

4   use of aequorin expressed fungi according to the

5   present invention, it can be seen that any

6   appropriate eukaryotic cell or organism could be

7   used (i.e. mammalian cells in place of the fungi)

8   which has been transformed with any appropriate gene

9   according to the present invention (i.e. halistaurin

10   in place of aequorin)

11

12   The examples refer to the following figures in

13   which;

14

15   Figure 16 shows a graph indicating the effect of 6

16   environmental samples on $[Ca^{2+}]_c$;

17

18   Figure 17 shows a graph indicating the effect of

19   ibuprofen analogue on $[Ca^{2+}]_c$;

20

21   Figure 18 shows a graph indicating the effect of

22   verpamil on $[Ca^{2+}]_c$;

23

24   Figure 19 is a table summarising the profiles of the

25   ibuprofen™ ((S)-(-)- o-Acetulmandelic acid) and

26   verapamil™ (Verapamil hydrochloride) analogues;

27

28   Figure 20 is a table summarising profiles of

29   cyclopiazonic acid (CPA) and KP4 (mycotoxin produced

30   by *Ustilago spp*);

31

34

1   Figure 21 is a graph showing the dose-dependent

2   effect of KP4 on the $[Ca^{2+}]_c$ response to 5 mM

3   external $CaCl_2$ (results represent mean ± SE);

4

5   Figure 22a is a graph showing the effect of known

6   antifungal drugs on $[Ca^{2+}]_c$ in *Aspergillus nidulans*;

7

8   Figure 22b is a graph showing the effect of known

9   antifungal drugs on $[Ca^{2+}]_c$ in *Aspergillus niger*;

10

11   Figure 22c is a graph showing the effect of known

12   antifungal drugs on $[Ca^{2+}]_c$ in *Aspergillus awamori*;

13   and

14

15   Figure 23 is a graph showing the effect of

16   amphotericin B on $[Ca^{2+}]_c$ (results represent mean ±

17   SE).

18

19   Figure 24 shows a graph showing the ffect of Cr^{6+} (5

20   min preincubation) on aequorin light emission in

21   response to the addition of external $CaCl_2$ (5 mM).

22   Results represent mean ± SE.

23

24   Figure 25 shows a graph showing the effect of Cr^{6+} (5

25   min preincubation) on $[Ca^{2+}]_c$ in response to the

26   addition of external $CaCl_2$ (5 mM). Results represent

27   mean ± SE.

28

29   General cytotoxicity

30   - pure chemicals and chemical mixtures can be tested

31   for their toxicity using aequorin-expressed fungi.

32   Procedure:

35

1   i. Add compound(s) of interest to fungus

2   ii. Monitor $[Ca^{2+}]_c$ for 5 min

3   iii. Then stimulate fungus with mechanical

4   perturbation, hypo-osmotic, hyper-

5   osmotic shock

6   iv. Monitor $[Ca^{2+}]_c$ for further 5 min

7

8   The parameter to be assessed is $[Ca^{2+}]_c$ final resting

9   level. If $[Ca^{2+}]_c$ resting level is still elevated

10   more then 50% after the 11 min measurements the

11   compound(s) are toxic. The level of toxicity can be

12   assessed by subsequent monitoring of $[Ca^{2+}]_c$ for

13   several hours. The longer the $[Ca^{2+}]_c$ concentration

14   is out of normal the more toxic the compound(s) are.

15   This way there is no need for complicated software

16   and this type of approach is ideally suitable for

17   binary answer, based on 1 parameter.

18

19   Figure 16 shows a graph indicating the effect of 6

20   environmental samples on $[Ca^{2+}]_c$. The graph

21   indicates that sample 006 is toxic as the $[Ca^{2+}]_c$

22   final resting level is increased by more than 150%

23   compared with the control.

24

25   Another parameter for the analysis of general

26   toxicity is the total amount of $[Ca^{2+}]_c$ emitted.

27   Based on this parameter it is very easy to build

28   dose response curves (see Fig. 21).

29

30   **High information multiparameters analysis**

31   - In cases when the binary answer is not sufficient

32   aequorin-based biosensor can produce much more

36

1　detailed data characterising not only the general

2　cytotoxicity but also penetrability (by analysing

3　the time between administration of the compound to

4　the point when $[Ca^{2+}]_c$ starts to increase) and modes-

5　of-action of the compounds (by comparing the profile

6　of $[Ca^{2+}]_c$ changes of the compound(s) of interest to

7　the library of profiles). If the mode-of-action of

8　the compound(s) of interest is unique and unknown

9　than the present invention can suggest whether the

10　compound(s) causes the permeabilization of the

11　membrane, opening of ion channels or the alteration

12　in behaviour of Ca^{2+} carriers. This approach is

13　ideally suitable for analysis of combinations of

14　compounds.

15

16　This approach can be used for both pollutants

17　monitoring as previously described but also for the

18　analysis of drug toxicity. e.g. ibuprofen and

19　verapamil. Figures 17 and 18 show the effect of

20　ibuprofen and verapamil analogues on the $[Ca^{2+}]_c$ and

21　the table shown in Figure 19 further summarises the

22　profiles of the ibuprofen and verapamil analogues.

23　**Profiling compounds of interest and creating the**

24　**libraries of fingerprints of compounds**

25　- The present invention is ideally suitable for

26　creating the library of profiles for certain

27　substances. These profiles are unique to a compound

28　with the particular mode-of-action. Also they are

29　unique to the strain of fungus used, which allows

30　creating very details and reproducible fingerprint

31　of a particular compound using the present

32　invention. The profiles can be created with

37

1 different physico-chemical stimuli (e.g. mechanical

2 perturbation, hypo-osmotic, hyper-osmotic shock,

3 cold shock, heat shock, pH shock). These

4 fingerprints can be programmed into the software and

5 any compounds or mixtures of interest can be

6 screened to match the desired fingerprint.

7

8 Procedure to create the fingerprint:

9 • Monitor initial $[Ca^{2+}]_c$ resting level for 1

10 min

11 • Add compound(s) of interest to fungus

12 • Monitor $[Ca^{2+}]_c$ for 5 min

13 • Then stimulate fungus with mechanical

14 perturbation, hypo-osmotic, hyper-osmotic

15 shock

16 • Monitor $[Ca^{2+}]_c$ for further 5 min

17 • Based on the data obtained the following

18 parameters can be quantified for each

19 $[Ca^{2+}]_c$ increase occurring during the

20 experiment.

21 Lag time

22 Rise time

23 Amplitude absolute

24 Amplitude relative

25 Length of transient (LT_{20}, LT_{50},

26 LT_{80})

27 Initial $[Ca^{2+}]_c$ level

28 Final $[Ca^{2+}]_c$ resting level

29 Recovery time

30 Total concentration of $[Ca^{2+}]_c$

38

1 • Above 6 steps can be performed on

2 different strains

3 • Compound can be tested at different

4 concentrations

5

6 Considering the nature of the experiment the minimum

7 number of parameters produced by one compound at a

8 particular concentration on one fungal strain is

9 equal 22.

10

11 **Analysis of food and drink products for the presence**

12 **of mycotoxins**

13 - Fungi transformed with aequorin gene can be also

14 used for the analysis of food and drink products for

15 the presence of mycotoxins since these toxins affect

16 $[Ca^{2+}]_c$. Examples of such effects are shown in Figure

17 20 where the effects of cyclopiazonic acid (CPA) and

18 KP4 (mycotoxins produced by *Ustilago spp*) are

19 summarised.

20

21 **Cosmetics safety testing**

22 - Since EU regulations forbid the use of animal

23 testing for cosmetics industry the manufacturers are

24 looking at the alternative methods to assess the

25 effect of new products. As the present invention is

26 ideally suited for analysis of not only pure

27 compounds but also their mixtures, it could be used

28 for analysis of the safety of novel cosmetic

29 products. The present invention is also ideal for a

30 long term monitoring of the effects of compounds (up

31 to 96 h), which therefore allows analysis of the

32 longer-term toxicity than bacterial biosensors. The

39

1 present invention is also suitable for use on
2 different substrates such as solid and liquid
3 supports.
4

5 **Identification of different fungal strains**
6 - It has been found that each particular compound
7 produces a different fingerprint when added to
8 different fungal species. This can be used to
9 diagnose the unknown fungus.
10

11 Procedure:

12 • The fungus can be either transformed with
13 the recombinant aequorin gene or can be
14 injected with the active aequorin.

15 • Then this fungus can be subjected to a
16 range of the antifungal drugs, profiles of
17 which have already been created.

18 • Obtained profiles can be compared with the
19 library of the fingerprints and this way
20 the fungal species can be identified.

21 Figures 22a, b and c show that 5 known antifungal
22 drugs (ketoconazole, clotrimazole, amphotericin B,
23 nystatin and filipin) caused a different $[Ca^{2+}]_c$
24 response in 3 different species of *Aspergillus* (*A.*
25 *nidulans, A. niger, A. awamori*).
26

27 **Optimisation of the current antifungal treatments**
28 - In order to administer drugs in the best possible
29 way it is important to determine no effect
30 concentration and dose response curves, and
31 frequency for administration of drugs. Also, in
32 view of the developing resistance of fungus and

40

1 other eukaryotes to currently available drugs,
2 clinicians are looking into using combination of
3 drugs. The present invention is ideally suitable for
4 such studies.
5
6 **Identification of compounds which would prevent**
7 **fungal growth on plastics, metals and other**
8 **materials**
9 – Since the present invention is suitable for long
10 term measurements it is possible to monitor the
11 development and growth of fungi on different
12 materials and plastics treated with different
13 agents. It is possible to monitor the state of
14 fungal physiology by subjecting the organism to
15 different physico chemical treatments and analysis
16 of the profiles obtained.
17
18 Although the invention has been particularly shown
19 and described with reference to particular examples,
20 it will be understood by those skilled in the art
21 that various changes in the form and details may be
22 made therein without departing from the scope of the
23 present invention.

41

1   **Claims**

2

3   1. A method of determining the presence of a

4   toxicant in a test sample, comprising the steps

5   of;

6   • exposing a eukaryote that has been

7   transformed with a light emitting Ca^{2+}

8   regulated photoprotein gene to a test sample

9   • measuring the light produced by the

10   transformed cell/organism

11   • determining whether the amount of light is

12   above or below a defined threshold at the

13   time of exposure.

14

15   2. A method as in Claim 1 wherein the eukaryote is a

16   fungi.

17

18   3. A method as in Claim 2 wherein the fungi is a

19   filamentous fungi.

20

21   4. A method as in Claims 2 or 3 wherein the fungi is

22   of the Aspergillus species.

23

24   5. A method as in Claim 1 wherein the eukaryote is a

25   mammalian cell.

26

27   6. A method as in Claim 1 wherein the eukaryote is a

28   plant cell.

29

30   7. A method as in any of the previous Claims wherein

31   the test sample comprises a toxicant.

32

42

1   8. A method as in any of the previous Claims wherein
2   the light emitting Ca^{2+} regulated photoprotein
3   gene is a recombinant gene.
4
5   9. A method as in any of the previous Claims wherein
6   the light emitting Ca^{2+} regulated photoprotein
7   gene is selected from the group comprising;
8   • aequorin gene
9   • halistaurin (mitrocomin) gene
10   • phialidin (clytin) gene
11   • obelin gene
12   • mnemiopsin gene
13   • berovin gene
14
15 10. A method as in any of the previous Claims
16   wherein the light emitting Ca^{2+} regulated
17   photoprotein gene may be a functional homologue
18   of a gene selected from the group comprising;
19   • aequorin gene
20   • halistaurin (mitrocomin) gene
21   • phialidin (clytin) gene
22   • obelin gene
23   • mnemiopsin gene
24   • berovin gene
25
26 11. A method as in any of the previous Claims
27   wherein the light emitting Ca^{2+} regulated
28   photoprotein gene is an aequorin gene.
29

43

1   12. A method as in any of the previous Claims
2   wherein the light emitting Ca^{2+} regulated
3   photoprotein gene is a recombinant aequorin gene.
4
5   13. A method as in any of the previous Claims
6   wherein the light that is measured is in the form
7   of luminescence.
8
9   14. A method as in any of the previous Claims
10   wherein the test sample is added in advance of
11   the application of a stimulus to the test sample.
12
13   15. A method as in Claim 14 wherein the stimulus is
14   at least one or more from the group comprising;
15   mechanical perturbation, hypo-osmotic shock,
16   change in external calcium chloride
17   concentration, temperature shock and pH shock.
18
19   16. A method as in Claims 14 and 15 wherein the
20   test sample is added 1 minute to 1 hour prior to
21   the application of the stimulus.
22
23   17. A method as in Claims 14 to 16 wherein the test
24   sample is added 5 minutes prior to the
25   application of the stimulus.
26
27   18. A method as in Claims 14 to 16 wherein the test
28   sample is added 30 minutes prior to the
29   application of the stimulus.
30

44

1 19. A method of determining the presence of a

2   toxicant in a test sample, comprising the steps

3   of;

4   • exposing a eukaryote that has been

5   transformed with a light emitting Ca^{2+}

6   regulated photoprotein gene to a test sample

7   • measuring the light produced by the

8   transformed cell/organism

9   • determining whether the amount of light is

10   above a defined threshold at a specified

11   time after the time of exposure.

12

13 20. A method as in Claim 19 which comprises the

14   step of determining whether the amount of light

15   is below a defined threshold.

16

17 21. A method as in Claims 19 and 20 wherein the

18   specified time after the time of exposure is 11

19   minutes.

20

21 22. A method as in Claims 19 to 21 wherein the

22   eukaryote is a fungi.

23

24 23. A method as in Claim 22 wherein the fungi is a

25   filamentous fungi.

26

27 24. A method as in Claims 22 to 23 wherein the

28   fungi is of the Aspergillus species.

29

30 25. A method as in Claims 19 to 21 wherein the

31   eukaryote is a mammalian cell.

32

45

1   26. A method as in Claims 19 to 21 wherein the

2   eukaryote is a plant cell.

3

4   27. A method as in Claims 19 to 26 wherein the test

5   sample comprises a toxicant.

6

7   28. A method as in Claims 19 to 27 wherein the

8   light emitting Ca^{2+} regulated photoprotein gene is

9   a recombinant gene.

10

11   29. A method as in Claims 19 to 28 wherein the

12   light emitting Ca^{2+} regulated photoprotein gene is

13   selected from the group comprising;

14   • aequorin gene

15   • halistaurin (mitrocomin) gene

16   • phialidin (clytin) gene

17   • obelin gene

18   • mnemiopsin gene

19   • berovin gene

20

21   30. A method as in Claims 19 to 29 wherein the

22   light emitting Ca^{2+} regulated photoprotein gene

23   may be a functional homologue of a gene selected

24   from the group comprising;

25   • aequorin gene

26   • halistaurin (mitrocomin) gene

27   • phialidin (clytin) gene

28   • obelin gene

29   • mnemiopsin gene

30   • berovin gene

31

46

1   31.   A method as in Claims 19 to 30 wherein the
2   light emitting Ca^{2+} regulated photoprotein gene is
3   an aequorin gene.
4
5   32.   A method as in Claims 31 wherein the light
6   emitting Ca^{2+} regulated photoprotein gene is a
7   recombinant aequorin gene.
8
9   33.   A method as in Claims 19 to 32 wherein the
10   light that is measured is in the form of
11   luminescence.
12
13   34.   A method as in Claims 19 to 33 wherein the test
14   sample is added in advance of the application of
15   a stimulus to the test sample.
16
17   35.   A method as in Claim 34 wherein the stimulus is
18   at least one or more from the group comprising;
19   mechanical perturbation, hypo-osmotic shock,
20   change in external calcium chloride
21   concentration, temperature shock and pH shock.
22
23   36.   A method as in Claims 34 to 35 wherein the test
24   sample is added 1 minute to 1 hour prior to the
25   application of the stimulus.
26
27   37.   A method as in Claims 34 to 36 wherein the test
28   sample is added 5 minutes prior to the
29   application of the stimulus.
30

47

1   38.   A method as in Claims 34 to 36 wherein the test

2   sample is added 30 minutes prior to the

3   application of the stimulus.

4

5   39.   A method of determining the presence of a

6   toxicant in a test sample, comprising the steps

7   of;

8   • exposing a eukaryote that has been

9   transformed with a light emitting Ca^{2+}

10   regulated photoprotein gene to a test sample

11   • measuring the light produced by the

12   transformed cell/organism

13   • and comparing the light measurement data

14   with a bank of known toxicity reference

15   data.

16

17   40.   A method as in Claim 39 wherein the method

18   comprises the step of determining whether the

19   amount of light is below a defined threshold.

20

21   41.   A method as in Claims 39 to 40 wherein the

22   specified time after the time of exposure is 11

23   minutes.

24

25   42.   A method as in Claims 39 to 40 wherein the

26   eukaryote is a fungi.

27

28   43.   A method as in Claim 42 wherein the fungi is a

29   filamentous fungi.

30

31   44.   A method as in Claims 42 to 43 wherein the

32   fungi is of the *Aspergillus* species.

48

1

2   45.   A method as in Claims 39 to 41 wherein the
3   eukaryote is a mammalian cell.

4

5   46.   A method as in Claims 39 to 41 wherein the
6   eukaryote is a plant cell.

7

8   47.   A method as in Claims 39 to 46 wherein the test
9   sample comprises a toxicant.

10

11 48.   A method as in Claims 39 to 47 wherein the
12   light emitting Ca^{2+} regulated photoprotein gene is
13   a recombinant gene.

14

15 49.   A method as in Claims 39 to 48 wherein the
16   light emitting Ca^{2+} regulated photoprotein gene is
17   selected from the group comprising;
18   • aequorin gene
19   • halistaurin (mitrocomin) gene
20   • phialidin (clytin) gene
21   • obelin gene
22   • mnemiopsin gene
23   • berovin gene

24

25 50.   A method as in Claims 39 to 49 wherein, the
26   light emitting Ca^{2+} regulated photoprotein gene
27   may be a functional homologue of a gene selected
28   from the group comprising;
29   • aequorin gene
30   • halistaurin (mitrocomin) gene
31   • phialidin (clytin) gene

49

1   • obelin gene

2   • mnemiopsin gene

3   • berovin gene

4

5   51. A method as in Claims 39 to 50 wherein the

6   light emitting Ca^{2+} regulated photoprotein gene is

7   an aequorin gene.

8

9   52. A method as in Claims 39 to 51 wherein the

10   light emitting Ca^{2+} regulated photoprotein gene is

11   a recombinant aequorin gene.

12

13   53. A method as in Claims 39 to 52 wherein the

14   light that is measured is in the form of

15   luminescence.

16

17   54. A method as in Claims 39 to 53 wherein the test

18   sample is added in advance of the application of

19   a stimulus to the test sample.

20

21   55. A method as in Claim 54 wherein the stimulus is

22   at least one or more from the group comprising;

23   mechanical perturbation, hypo-osmotic shock,

24   change in external calcium chloride

25   concentration, temperature shock and pH shock.

26

27   56. A method as in Claims 54 to 55 wherein the test

28   sample is added 1 minute to 1 hour prior to the

29   application of the stimulus.

30

50

57. A method as in Claims 54 to 56 wherein the test sample is added 5 minutes prior to the application of the stimulus.

58. A method as in Claims 54 to 55 wherein the test sample is added 30 minutes prior to the application of the stimulus.

59. A method as in Claims 39 to 58 wherein the method is used to determine the amount of toxicant in the sample.

60. A method as in Claims 39 to 59 wherein the method is used to identify the toxicant in the sample.

61. A method of determining the presence of a toxicant in a test sample, comprising the steps of;

- exposing a eukaryote that has been transformed with a light emitting Ca^{2+} regulated photoprotein gene to a test sample
- measuring the light produced by the transformed cell/organism
- converting the light data into a cytosolic free calcium ion concentration trace,
- and comparing at least one parameter of the cytosolic free calcium ion concentration trace with a bank of known toxicity reference data.

51

62. A method as in Claim 61 wherein the method comprises the step of determining whether the amount of light is below a defined threshold.

63. A method as in Claims 61 to 62 wherein the eukaryote is a fungi.

64. A method as in Claim 63 wherein the fungi is a filamentous fungi.

65. A method as in Claims 63 to 64 wherein the fungi is of the *Aspergillus* species.

66. A method as in Claims 61 to 62 wherein the eukaryote is a mammalian cell.

67. A method as in Claims 61 to 62 wherein the eukaryote is a plant cell.

68. A method as in Claims 61 to 67 wherein the test sample comprises a toxicant.

69. A method as in Claims 61 to 68 wherein the light emitting Ca^{2+} regulated photoprotein gene is a recombinant gene.

70. A method as in Claims 61 to 69 wherein the light emitting Ca^{2+} regulated photoprotein gene is selected from the group comprising;
    • aequorin gene
    • halistaurin (mitrocomin) gene
    • phialidin (clytin) gene

52

1   • obelin gene

2   • mnemiopsin gene

3   • berovin gene

4

5   71.   A method as in Claims 61 to 70 wherein the

6   light emitting Ca^{2+} regulated photoprotein gene

7   may be a functional homologue of a gene selected

8   from the group comprising;

9   • aequorin gene

10   • halistaurin (mitrocomin) gene

11   • phialidin (clytin) gene

12   • obelin gene

13   • mnemiopsin gene

14   • berovin gene

15

16   72.   A method as in Claims 61 to 71 wherein the

17   light emitting Ca^{2+} regulated photoprotein gene is

18   an aequorin gene.

19

20   73.   A method as in Claims 61 to 72 wherein the

21   light emitting Ca^{2+} regulated photoprotein gene is

22   a recombinant aequorin gene.

23

24   74.   A method as in Claims 61 to 73 wherein the

25   light that is measured is in the form of

26   luminescence.

27

28   75.   A method as in Claims 61 to 74 wherein the test

29   sample is added in advance of the application of

30   a stimulus to the test sample.

31

53

76. A method as in Claim 75 wherein the stimulus is
at least one or more from the group comprising;
mechanical perturbation, hypo-osmotic shock,
change in external calcium chloride
concentration, temperature shock and pH shock.

77. A method as in Claims 75 to 76 wherein the test
sample is added 1 minute to 1 hour prior to the
application of the stimulus.

78. A method as in Claims 75 to 77 wherein the test
sample is added 5 minutes prior to the
application of the stimulus.

79. A method as in Claims 75 to 77 wherein the test
sample is added 30 minutes prior to the
application of the stimulus.

80. A method as in Claims 61 to 79 wherein light is
measured for between 1 minute and 5 hours
following the application of the stimulus.

81. A method as in Claims 61 to 79 wherein light is
measured for between 1 minute and 96 hours
following the application of the stimulus.

82. A method as in Claims 61 to 79 wherein light is
measured for 5 minutes following the application
of the stimulus.

83. A method as in Claims 61 to 82 wherein the
cytosolic free calcium ion trace is a plot of the

54

1   cytosolic free calcium ion concentration against

2   time.

3

4   84.   A method as in Claims 61 to 83 wherein the

5   parameter is at least one or more selected from

6   the group comprising;

7   • lag time

8   • rise time

9   • absolute amplitude

10   • relative amplitude

11   • Length of transient at 20%, 50% and 80% of

12   maximum amplitude

13   • number of cytosolic free calcium ion

14   concentration increases

15   • percentage increase in final cytosolic free

16   calcium ion concentration resting level

17   • percentage increase in recovery time

18   • percentage increase in pre-stimulating

19   cytosolic free calcium ion concentration

20   resting level

21   • total concentration of calcium

22

23   85.   A method as in Claims 61 to 84 wherein the

24   method is used to determine the amount of

25   toxicant in the sample.

26

27   86.   A method as in Claims 61 to 85 wherein the

28   method is used to identify the toxicant in the

29   sample.

30

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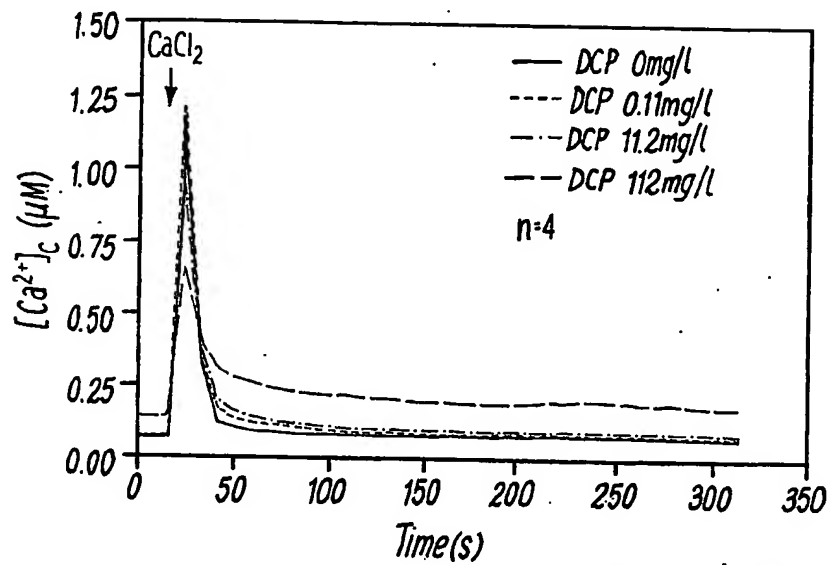
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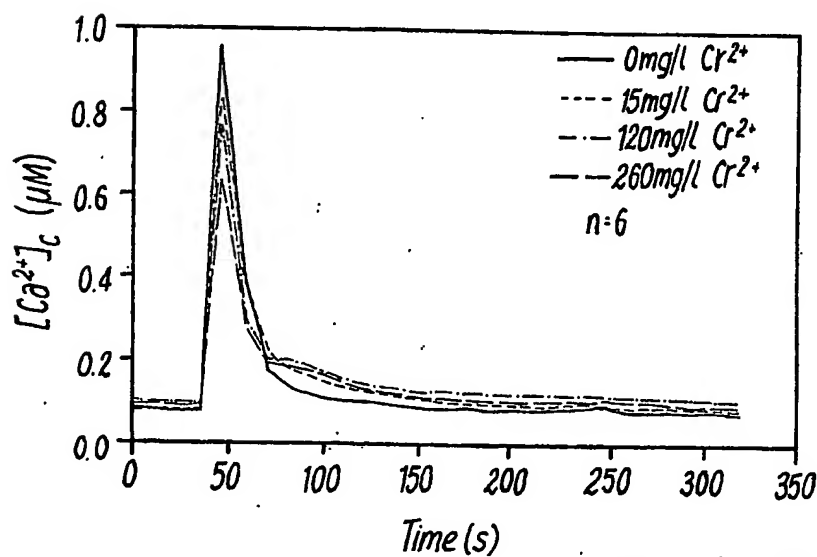
**Fig.2**

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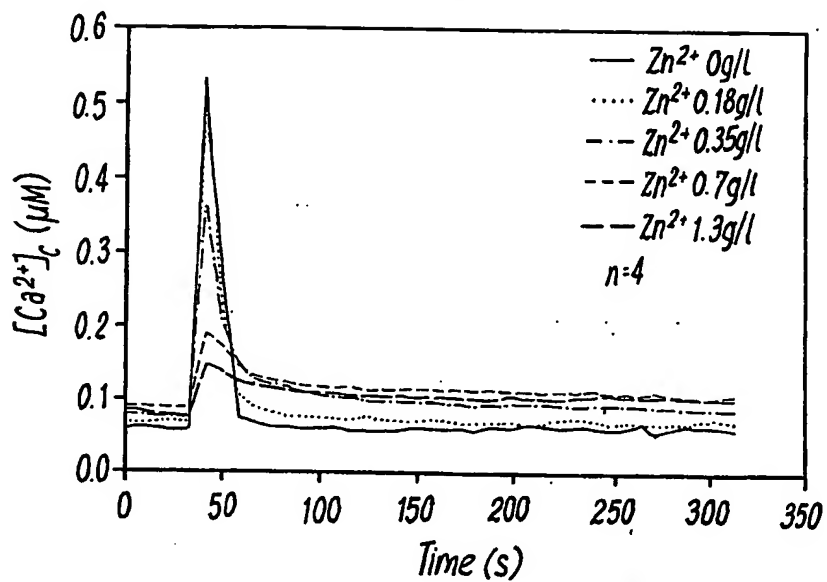
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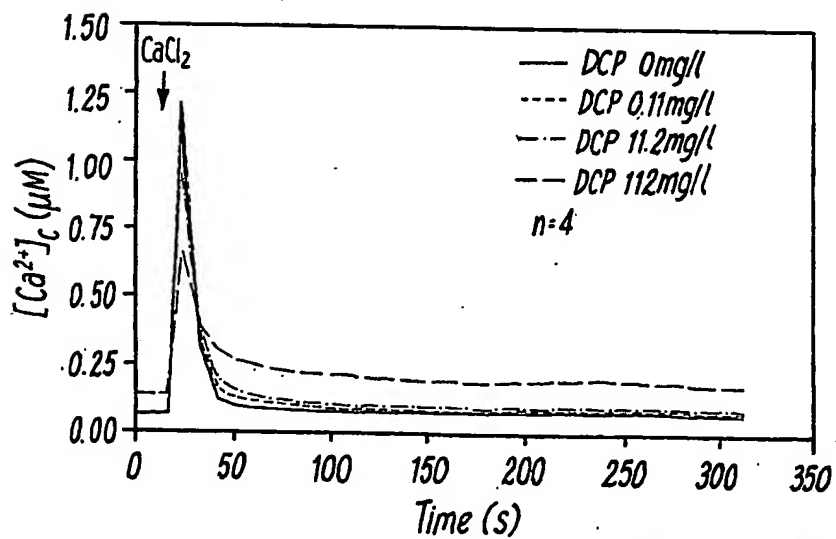
Fig.4

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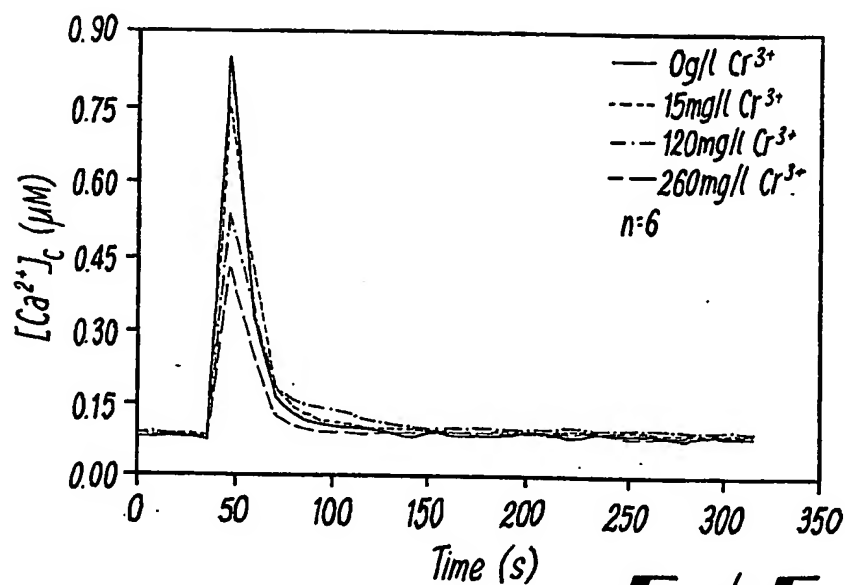
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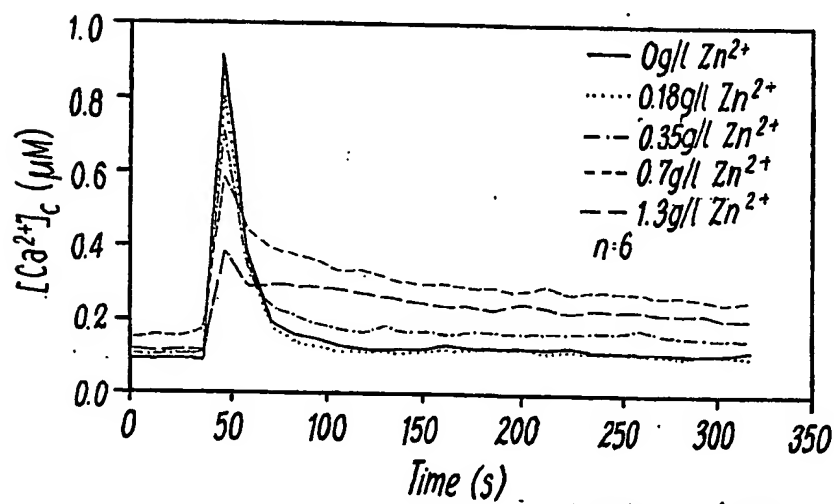
Fig. 6
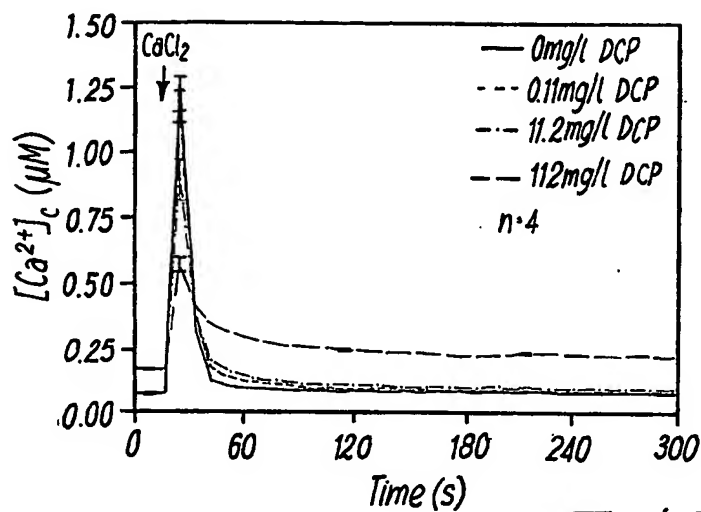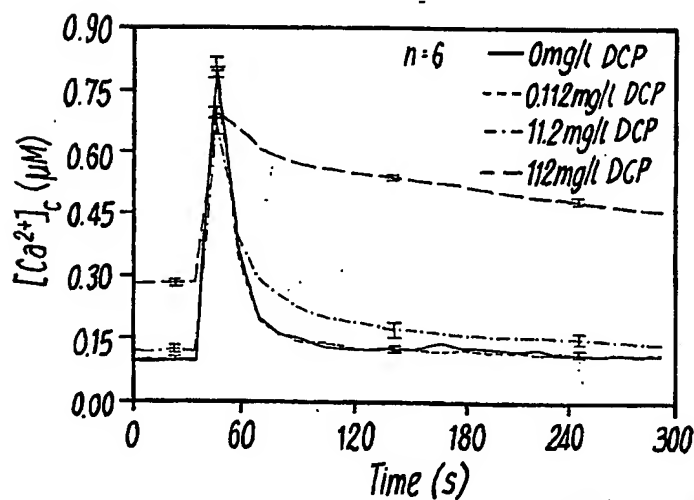
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FIG. 7

FIG. 8

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Fig. 9

Fig. 10

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Fig. 11

Fig. 12

FIG. 13
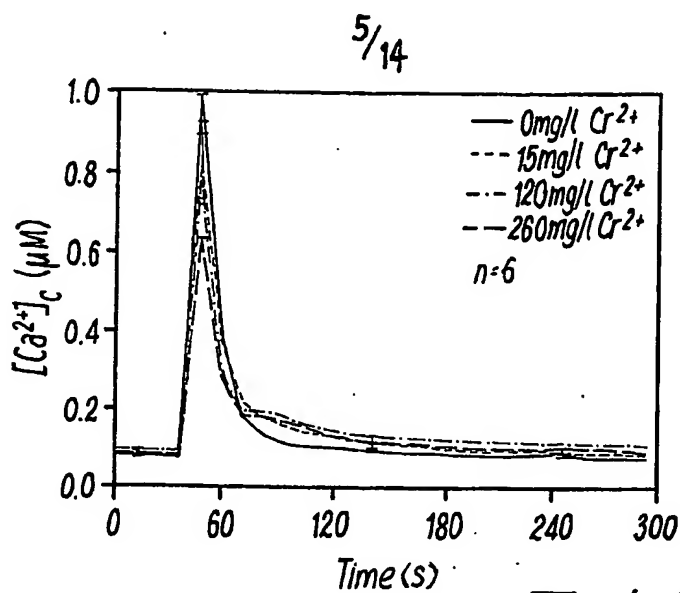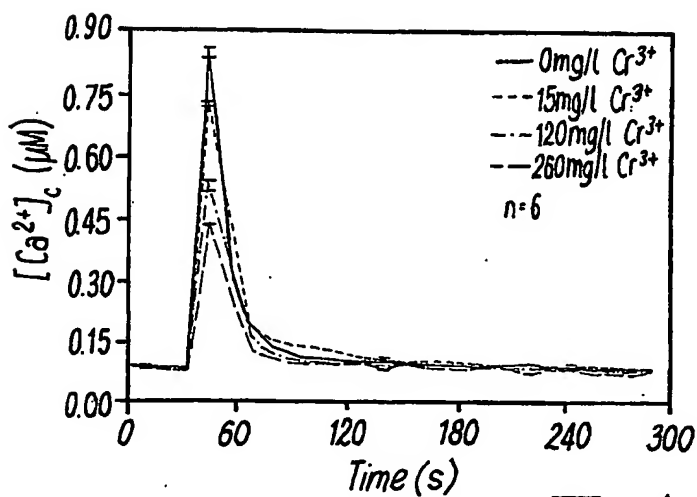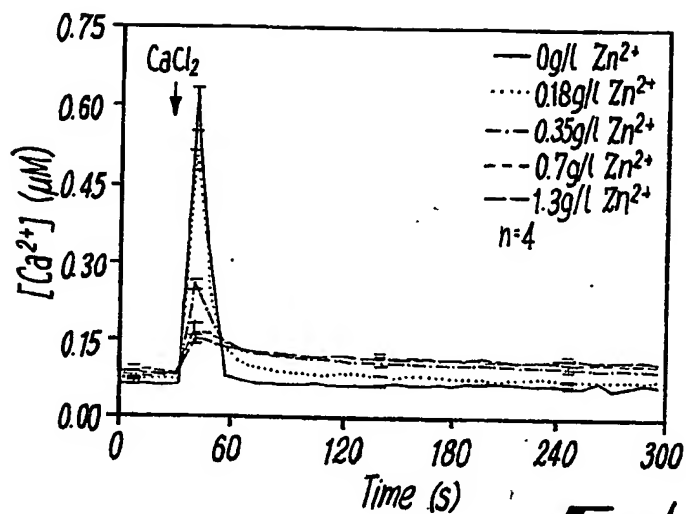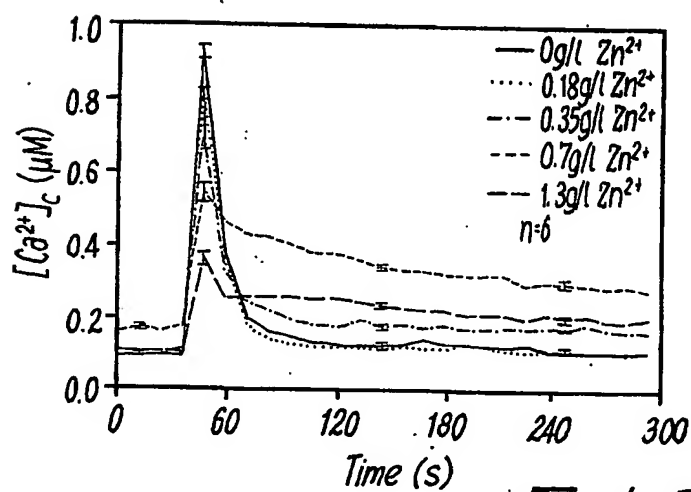
Chemical	Values of interest (mg/l)	LT		RT		A		LT$_{50}$		%IpreSRL	%IFRL		%IRT		Number of increases	
		S1	S2	S1	S2	S1	S2	S1	S2	S2	S1	S2	S1	S2	S1	S2
PCP	0.01	0	0	1	1	75±23	100±5	↑	-	97±9	115±6	104±6	111±4	102±3	1	1
	0.1	0	0	1	1	74±22	91±5	↑	-	104±9	118±5	104±11	122±9	99±3	1	1
	1	0	0	1	1	72±14	80±10	↑	-	109±5	204±13	107±11	195±8	109±6	1	1
	5	0	0	12.6	1	199±26	89±14	↑	↑	120±3	209±7	205±9	274±4	218±7	1	1
	10	0	0	12.6	12.6	417±73	175±27	↑	↑	131±9	305±17	253±7	373±22	308±4	1	1
SDS	1	0	0	1	1	111±48	102±8	-	-	125±34	116±5	106±7	114±6	109±9	1	1
	10	0	0	12.6	1	246±19	118±4	↑	↑	136±13	162±7	120±13	243±14	154±10	1	1
	50	0	0	12.6	1	295±33	115±12	↑	↑	323±19	237±9	222±16	328±6	287±10	1	1
	100	0	0	12.6	1	293±19	116±3	↑	↑	359±24	405±15	256±14	367±11	286±12	1	1
	500	0	n.a.	12.6	n.a.	998±35	n.a.	↑	n.a.	n.a.	561±12	n.a.	565±12	n.a.	1	n.a.
Toluene	1	n.a.	0	n.a.	1	n.a.	74±13	n.a.	-	129±9	n.a.	113±3	n.a.	115±2	n.a.	1

Note: LT=lag time
RT=rise time
A=changes in A (%)

%IpreSRL=% increase in pre-stimulation resting level
%IFRL=% increase in final resting level
%IRT=% increase in recovery time
S1=Stage 1
S2=Stage2

Chemical	Values of interest (mg/l)	LT S1	LT S2	RT S1	RT S2	A S1	A S2	LT$_{50}$ S1	LT$_{50}$ S2	%IpreSRL S2	%IFRL S1	%IFRL S2	%IRT S1	%IRT S2	Number of increases S1	Number of increases S2
3,5 DCP	10	0	0	12.6	1	40±2	79±25	↑	↑	102±3	134±10	172±21	153±13	207±23	1	1
PCP	10	0	0	12.6	12.6	417±73	175±27	↑	↑	131±9	305±17	253±7	373±22	308±4	1	1
Zn^{2+}	700	0	0	12.6	1	42±5	74±1	↑	↑	142±18	225±8	263±14	258±5	225±12	1	1
Cr^{6+}	15	0	0	1	1	41±5	84±23	-	-	102±12	104±5	110±11	103±2	120±28	1	1
Toluene	25 (1)	0	0	n.a.	1	n.a.	73±13	n.a.	↑	121±6	n.a.	113±3	n.a.	115±2	1	1
SDS	500	0	0	12.6	1	998±35	116±3	↑	↑	359±24	561±12	256±14	565±12	286±12	1	1

Note: LT=lag time
     RT=rise time
     A=changes in A (%)

%IpreSRL=% increase in pre-stimulation resting level
%IFRL=% increase in final resting level
%IRT=% increase in recovery time
S1=Stage 1
S2=Stage2

FIG. 14

| Chemical	Values of interest (mg/l)	LT S1	LT S2	RT S1	RT S2	A S1	A S2	LT50 S1	LT50 S2	%IpreSRL S2	%IFRL S1	%IFRL S2	%IRT S1	%IRT S2	Number of increases S1	S2
3,5 DCP	10	0	0	12.6	1	40±2	79±25	↑	↑	102±3	134±10	172±21	153±13	207±23	1	1
Cr⁶⁺	15	0	0	1	1	41±5	84±23	-	-	102±12	104±5	110±11	103±2	120±28	1	1
Zn²⁺	700	0	0	12.6	1	42±5	74±1	↑	↑	142±18	225±8	263±14	258±5	225±12	1	1
SDS	500	0	0	12.6	1	998±35	116±3	↑	↑	359±24	561±12	256±14	565±12	286±12	1	1
3,5-DCP + Cr⁶⁺	6+12	0	0	12.6	1	33±4	88±13	↑	-	96±26	117±4	120±14	128±5	119±10	1	1
Cr⁶⁺ Zn²⁺	30+350	0	0	1	1	23±6	76±4	↗	-	100±5	143±18	150±12	158±26	154±6	1	2
3,5DCP +Zn²⁺	10+350	0	0	12.6	1	65±5	86±2	↗	-	103±9	294±18	153±16	311±14	208±11	1	2
3,5-DCP+ Cr⁶⁺ Zn²⁺	6+12+350	0	0	12.6	1	25±2	79±5	↗	-	102±8	164±4	150±9	160±5	195±8	1	2
Mixture 1	See M&M	0	0	12.6	12.6	466±13	128±6	↑	↑	262±13	402±17	501±38	446±17	477±28	2	2
Mixture 2	See M&M	0	0	1	1	116±8	69±14	↑	-	148±16	177±44	132±27	170±28	120±7	2	1

Italics represents data obtained with very high concentrations of toxicants: Zn^{2+}=700 mg/l; Cr^{6+}=120 mg/l; 3,5 DCP=49 mg/l

FIG. 15

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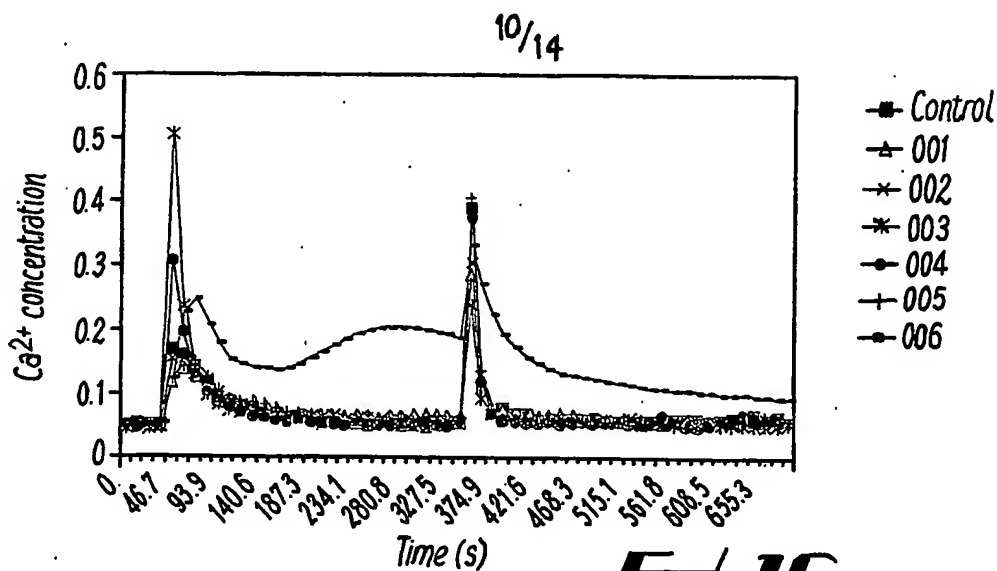
FIG. 16
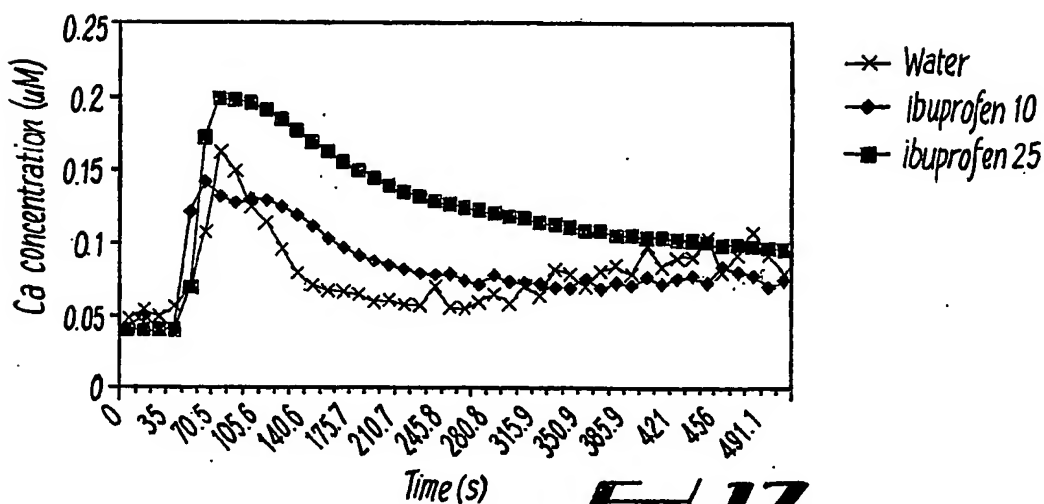
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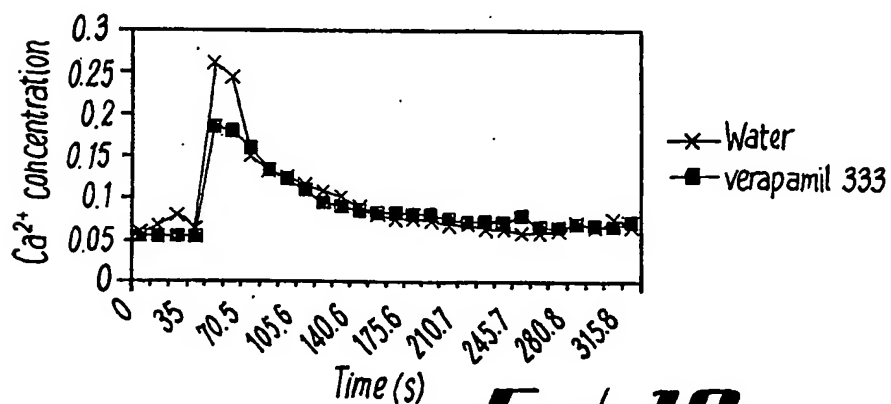
FIG. 18

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$^{11}/_{14}$

Chemical	Concentration (mg/l)	RT	A	LT$_{50}$	%IFRL	%IRT
Ibuprofen	10	↓	-	↓	-	-
	25	-	↑↑	↑	-	↑
Verapamil	333	-	↑	-	-	-

Fig.19

Chemical	Concentration (μM)	RT	A	LT$_{50}$	%IPreRL	%IFRL	%IRT
CPA	10	-	-	-	-	-	-
	20	-	↑	↑	↑	↑	↑
	50	-	↑↑	↑	3*↑	4*↑	4*↑
KP4	5.4	-	↓	↑	-	-	-

Fig.20
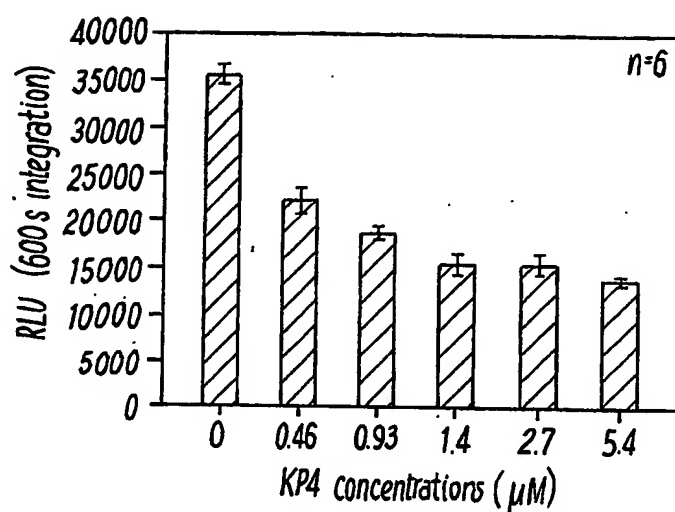
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FIG.21

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Aspergillus nidulans

Legend:
- ■ Control
- ▲ ketoconazole
- ✳ clotrimazole
- --- amphotericin B
- △ nystatin
- ◆ filipin

Fig.22a

Aspergillus niger

Legend:
- ■ Control
- ▲ ketoconazole
- ✳ clotrimazole
- --- amphotericin B
- △ nystatin
- ◆ filipin

Fig.22b

Aspergillus awamori

Legend:
- ■ Control
- ▲ ketoconazole
- ✕ clotrimazole
- ── amphotericin B
- △ nystatin
- ◆ filipin

Fig.22c

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FIG. 23

FIG. 24

FIG. 25

SUBSTITUTE SHEET (RULE 26)
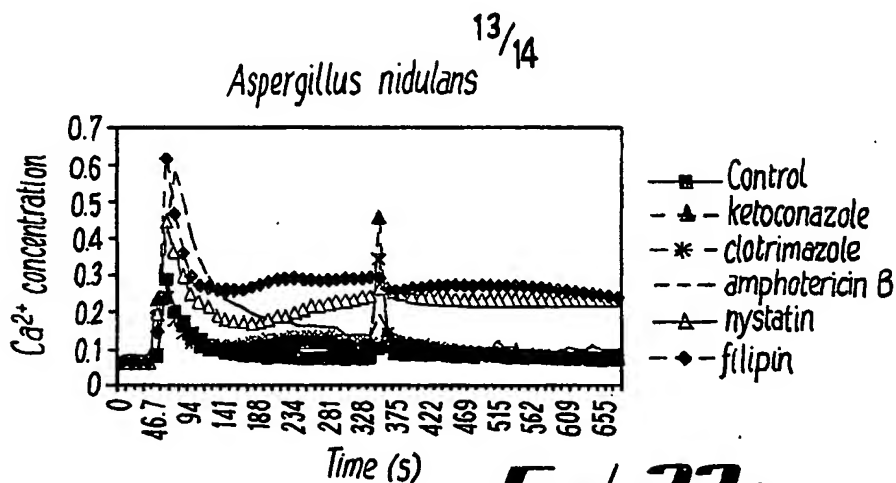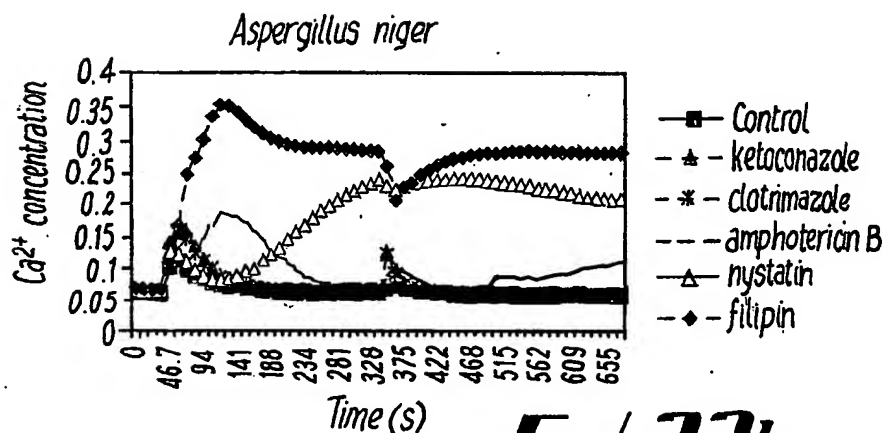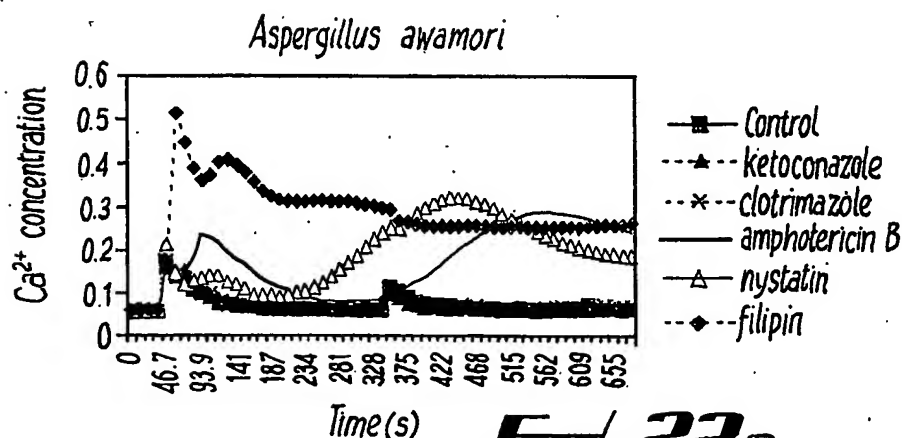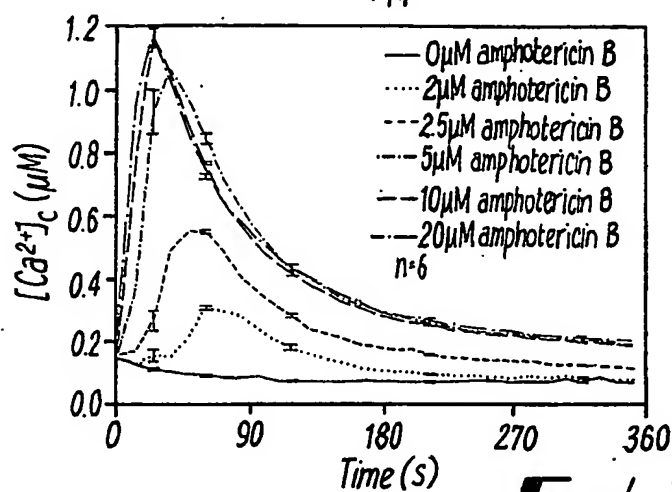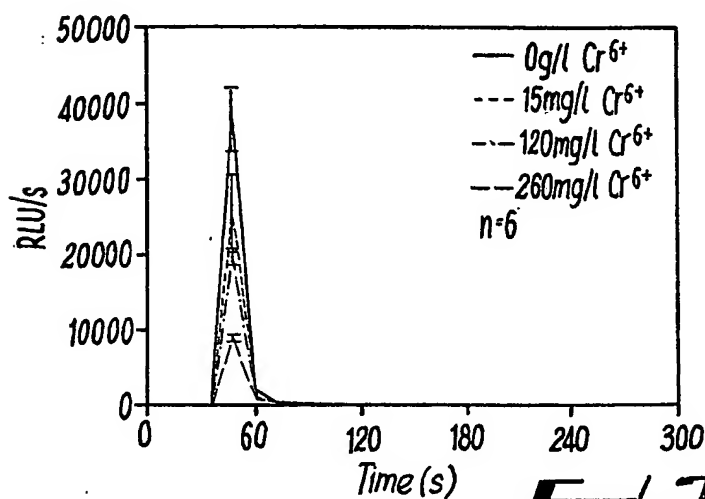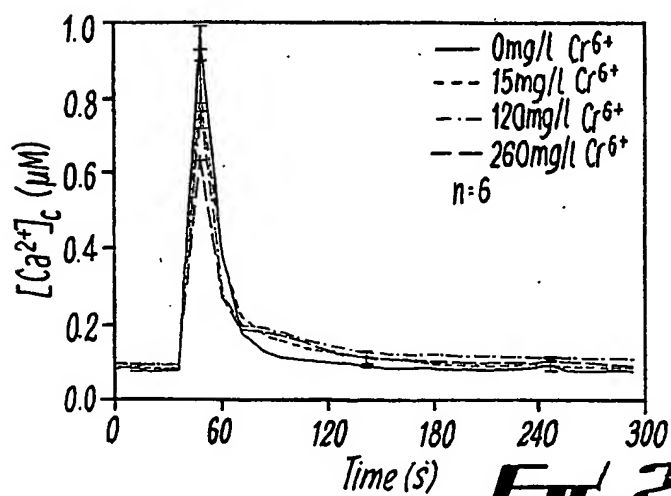
WO 2004/050905 **NATIONAL SEARCH REPORT**

Internal	PCT/GB2003/005272
PCT/.. 03/05272	

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7   C12Q1/66   C12Q1/68

Rec'd PCT/PTO 03 JUN 2005

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
IPC 7   C12Q
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 00/02045 A (DETHEUX MICHEL ; DUPRIEZ VINCENT (BE); EUROSCREEN S A (BE); PARMENTIER) 13 January 2000 (2000-01-13) page 10; example 1 page 13; example 5 figures 2,6	1-60
X	WO 02/075273 A (DUPRIEZ VINCENT ; EUROSCREEN S A (BE); PARMENTIER MARC (BE)) 26 September 2002 (2002-09-26) abstract page 30; example 1 page 35; examples 2,3 page 29, lines 5-10 claims 1-3; figures 2,11	1-60
	—/—	

[X]	Further documents are listed in the continuation of box C.		[X]	Patent family members are listed in annex.

* Special categories of cited documents :

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16 March 2004	07/04/2004

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Leber, T

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INTERNATIONAL SEARCH REPORT

Internal Application No

PCT/ᵤᵤ J3/05272

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
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